DEVELOPMENT OF A LOW-COST, PORTABLE ASSAY TO GENOTYPE STRAINS OF THE AMPHIBIAN-KILLING FUNGUS *BATRACHOCHYTRIUM DENDROBATIDIS*

HONORS THESIS

Presented to the Honors College of
Texas State University
in Partial Fulfillment
of the Requirements

for Graduation in the Honors College

by

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San Marcos, Texas
May 2018

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ACKNOWLEDGEMENTS

Thank you, Dr. David Rodriguez, Dr. Dittmar Hahn, the RDZ Lab, Texas State Honors College, Ms. Selena Overton, family, and friends. I especially thank Gabriela Solis for training and assistance in the lab. I thank Quentin DiPasquale, Phuong Ngoc Minh Le, and Gabriela Solis for reviewing an earlier draft of this thesis.

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LIST OF ABBREVIATIONS

ARMS- Amplification Refractory Mutation System

Bd- Batrachochytrium dendrobatidis

Bsal- Batrachochytrium salamandrivorans sp. nov.

DNA- Deoxyribonucleic acid

EID- Emerging Infectious Disease

ExoSAP- Exonuclease Shrimp Alkaline Phosphotase

MM- Master Mix

SNPs- Single Nucleotide Polymorphisms

PCR- Polymerase Chain Reaction

GPL- Global Panzootic Lineage

ABSTRACT

With the rise of the fungal pathogens like amphibian chytrids, amphibian species across the world are threatened and some populations are dwindling at an alarming rate. Increased genetic sampling of amphibian chytrids has shown more genetic diversity that was previously known. Although there are methods to identify strains by genotyping, these techniques usually involve costly laboratory equipment that is impractical in remote regions such as Ecuador, a region of high amphibian diversity. This project focuses on an efficient and low-cost method of genotyping two different strains, *Bd*-GPL and *Bd*-Brazil, using tetra-primer PCR and custom-designed genotyping assays. These assays comprise of an external PCR and then genotype specific internal PCRs. Thus far, external PCRs have been successful across several local strains of *Bd*, and work on the genotype specific PCRs is ongoing. In the next phase, this assay will be tested on samples collected from Ecuador, and it will be optimized for deployment in the field using portable equipment.

I. Introduction to Chytrids

The world of fungi, diverse and mysterious in its own right, has had a significant role in the ecological and evolutionary paths of bacteria, plants, animals, and other fungi. Although there are more well-known, fruiting forms of fungi such as *Agaricus muscarius*, commonly known as the famous "Fly Agaric", there is much more to the field of Mycology that impacts the well-being of humans, animals, and plants. Aside from the fleeting phase which functions as the dispersal of spores, there is a lifetime spent underground (or on a preferred substrate) as hyphae, a thread-like component of mycelium which functions as a network and is a vegetative form of fungi. These organisms, not only prone to act as saprobes within the environment, may also have the capability of parasitizing live hosts. Within the last 15 years, scientists have recently begun researching the globally distributed and recently emerged amphibian-chytrid, *Batrachochytrium dendrobatidis* (*Bd*), which has caused sharp decline in some populations of amphibians across the globe and is known as an Emerging Infectious Disease (EID). The epidemiology of *Bd* is complex, its origins and the reason for its sudden shift towards pathogenicity are unknown and are thought to be an accidental occurrence.

Bd, different from its fungal relatives, is a posterior flagellated, single-celled organism with an unknown origin and sexual reproductive cycle. The asexual cycle, however, is completed through the use of zoospores (a motile spore with the use of flagellum). Like all true Fungi (identified in literature by a capitalized F), *Bd* contains chitin, makes use of the AAA-lysine biosynthetic pathway, and uses glycogen as a storage carbohydrate. With nearly most global domains at its disposal, chytrid is usually

found in aquatic environments including acidic bogs and can also thrive in terrestrial environments. As chytridiomycosis (disease caused by *Bd*) manifests throughout the amphibian organism, beginning with the keratinized portion of tadpole lips, it causes disruption of equilibria by thickening of the skin, hindering electrolyte function, gas exchange, and skin/tissue structure. The development of *Bd* within its host will eventually lead to death and can further spread to other susceptible individuals within a population (Rosenblum et al., 2013).

Batrachochytrium dendrobatidis has the ability to infect nearly 500 amphibian host species with various factors coming into play with amphibian susceptibility (James et al., 2015). Some species of frogs however, are asymptomatic to chytridiomycosis and show no negative effects from infection. These frogs can act as reservoirs that aid in the spread and distribution of *Bd* (Briggs et al., 2010). Recent globalization has made transportation easier, and thus has also created a "New Pangea," effect which increases the chance of naïve species having first contact with foreign organisms.

Furthermore, greater diversity within the phylum: Chytridiomycota is being detected as sampling has increased. Chytridiomycosis was thought to only occur in frogs and toads, but a second chytrid, *Batrachochytrium salamandrivorans* sp. nov. has caused sudden sharp decline in fire salamander (*Salamandra salamandra*) populations; the once thriving species has now dwindled down to a mere 4% of what it had originally been in the Netherlands. Within the *Batrachochytrium dendrobatidis* species group, the two main strains of *Batrachochytrium dendrobatidis* (*Bd*-GPL and *Bd*-Brazil) exhibit the most diversity amongst relative *Bd* species, are prevalent in the Americas, and are known to cause devastating and lethal chytridiomycosis in amphibians in comparison to other

species. The divergence between these strains is hypothesized to have originated ~104,700 years ago, whereas *Bd*-GPL1 and *Bd*-GPL2, the dominant strains causing major chytridiomycosis outbreaks in the Americas, are estimated to have diverged from a common ancestor ~26,400 years ago (Rosenblum et al., 2013). Although, the evolutionary history of *Bd* is complex and is recognized as both novel and endemic depending on the region of study.

Polymerase Chain Reaction (PCR), is a laboratory technique commonly used to amplify small amounts of DNA through a range of temperatures that facilitate strand denaturation, primer annealing and DNA synthesis. Components include the DNA template that one wishes to amplify, DNA Polymerase, an enzyme that can withstand high temperatures and has the ability to synthesize a new complimentary strand of DNA, Nucleotides, monomers of nucleic acids, and finally primers, which direct elongation of DNA in accordance to the complimentary makeup of the original DNA strand. In a PCR reaction, forward and reverse primers bind to either the 3' or 5' strand of DNA at opposite sides during amplification, which results in complimentary DNA fragments of varying sizes depending on primer pairs. A common problem faced in PCR is the mispriming, which results in spurious bands shown in products (Don et al., 1991). To avoid this, common techniques such as making Mg^{2+} adjustments or using a touchdown PCR, have proven to be efficient. Touchdown PCR increases specificity by starting the reactions relatively high temperatures (higher specificity), and slowly lowers it to ~55°C, the minimal annealing temperature ~55°C (lower specificity). Another PCR technique which enhances specificity is a gradient PCR which runs reactions under a range of annealing temperatures. The gradient aids in determining optimal annealing temperatures

by comparing the reactions by temperature. Products are then visible through gel electrophoresis.

Single nucleotide polymorphisms (SNPs) are known as single base pair mutations within a DNA. They make up more than 90% of variation within humans and can be used as genetic markers that may be useful in identifying genetic diversity. In the instance that one is comparing strains of chytrid, a SNP could be diagnostic for specific strains (Ye, 2001). In this project, the program http://primer1.soton.ac.uk/primer1.html was easily accessed and navigated for the design of primer assays which are accessible through the internet. This primer designing software is able to produce tetra-primer ARMS-PCR that functions as a single PCR assay which requires no post modification, thus this method is more efficient and less expensive than other alternatives (Collins, 2012). Assays may easily be designed with a known source sequence of DNA, SNP position, and alleles. Alongside the benefit of simple and easy design, users are able to modify annealing temperatures, GC content, and salt concentration, all of which are important factors to a successful PCR. Prior to designing assays, DNA may be analyzed through the bioinformatics computer software, Geneious, which allows users to layout DNA sequence data, locate SNP positions, and even test primers for functionality (Kearse et al., 2012).

Through the process known as electrophoresis, amplicons from PCRs are separated based on their length and weight by running an electric current through the agarose medium in which the DNA is inserted into. Because DNA has a slight negative charge, it is attracted towards positive charges. The design of the gel electrophoresis allows DNA to run towards the positive charge through an agarose gel (typically 1-3%

agarose) and TBE buffer to carry electric current through the apparatus. Due to the primers attaching to offset sites compared to the SNP position, fragments from the tetra-primer PCR will vary in length on a gel depending on genotype. Differentiation between bands is possible by comparison to the DNA ladder, which aids in the molecular-weight estimation of DNA.

Dr. David Rodriguez has developed a method of genotyping strains of *Bd (Bd*-GPL and *Bd*-Brazil) using quantitative PCR and digital PCR, but he will be traveling to remote areas of Ecuador that do not have access to the equipment needed to use these methods. The development of a lower-cost, portable method is necessary so that genotyping may be performed without the need of expensive lab equipment and at the sampling locations, which may only have basic or no lab facilities. This opens doors and creates opportunities to sample other locations around the world that may not have access to laboratory resources or viable internet connection in rural areas. The goal of this project is to create a portable PCR-based assay to genotype *Bd* in Ecuador.

II. Materials and Methods

The Primer1 website was able to provide the user with several outputs of primer assays; based on primer length, annealing temperature and other factors, the user is able to choose between ideal primer assays. Based on their sequence, primers will anneal to the complimentary portion of the DNA sequence in order to synthesize new fragments of DNA. Depending on the forward and reverse primer used in the PCR reaction, fragments of varying sizes are visibly shown through gel electrophoresis. The external primers (Fouta1a7 and Routa1a7) are expected to synthesize the longest fragments in comparison

to the internals (FinC and RinA) and the external primer they are paired with (Fouta1a7 and Routa1a7) (**Figure 1**).

To create a 100-µM stock solution for each primer, primers were centrifuged for no longer than 3 seconds, resuspended with respective amounts of nuclease-free H_2O into the original primer centrifuge tube by multiplying values by 10 (**Table 1**), vortexed for 15 seconds and allowed to rest at room temperature overnight. For a 10-µl solution, 60-µL of stock solution was transferred to new, labeled centrifuge tubes along with 540-µL of nuclease free water (3 tubes each primer). Each tube was then vortexed for 15 seconds and put into a freezer for storage. The stock solutions were stored in a stock primer box under the same conditions.

For the Touchdown Polymerase Chain Reaction (PCR), (4) 8-tube strips were labeled 1-8 along with respective master mix number. Master mixes (MM) were compromised of Nuclease-free H_2O, respective forward and reverse primers, DreamTaq 2X MM (ThermoFisher Scientific), $MgCl_2$, and BSA (400 ng/µL); each was transferred to respective strips in the same amounts (**Table 2**). After reaction master mixes were complete, seven samples and a negative control were transferred to each strip with their respective master-mix from tubes 1-8, finally the touchdown PCR was set to 65-60°C for 33 cycles in the Eppendorf MasterCycler PCR machine (**Table 3**). When PCRs were complete, a 2% gel was created by weighing 1.0 g of agarose in 50-mL of 1X TBE buffer in an Erlenmeyer flask, microwaved for ~30 seconds or until solution was clear, and allowed to cool (still liquefied) for pouring in a gel cast with combs in place. Once the gel cast was solidified and combs were carefully removed, the gel was then transferred to an electrophoresis apparatus with the well near the black, negatively charged electrode. The

DNA will run towards the positively charged electrode because negatively charged DNA will be forced to travel to the cathode. Products may be seen on **Figure 2A and 2B**. After analyzing results, another PCR reaction was conducted to test internal primers and externals (**Table 4 and Table 5**) Products are as seen on **Figure 3**.

For gradient PCR, the protocol was similar to previous PCR protocol, but with different PCR settings (gradient) on the MasterCycler PCR machine and only used sample TM33 (*Bd* isolated from Texas) in all reactions. Gel electrophoresis protocol and agarose remained the same as above. Reagents and volumes of reactions may be seen on **Table 6** and products on **Figure 4**.

To hydrolize primer dimers and any excess components of PCR (free primers and nucleotides) that will lower the quality of gel electrophoresis, samples were cleaned through ExoSAP protocolby the creation of a master-mix containing 1.56-µL of nuclease-free H_2O, 0.04-µL exonuclease and 0.40-µL SAP. Each reaction contained 2.0-µL of MM and 5.0µL PCR product and was inserted into the MasterCycler under an ExoSAP-specific program. Further testing using the ExoSAP products were carried through with a multiplex gradient was ensued with sample TM33's ExoSAP products. Contents and volumes may be seen on **Table 5** and products on **Figure 5**. The gradient was set to a range of 55-65℃ for 35 cycles.

III. Results/Discussion

Touchdown PCR Results

The 65-60°C touchdown PCR proved all primers worked adequately. Master-mix 1 and master-mix 4 had the best banding with samples TM26, TM33, and JEL423 (for MM1)

due to externals producing larger fragments. After the two gels were analyzed, it was then decided that more stringent meausures were necessary in order to get stronger readings on the internal primer fragments.

Allele-Specific and External (Routa1a7) Primer Testing

These PCR reactions were carried through with the external Routa1a7. With master-mix 1 (FinC and Rout_a1a7) and master-mix 2 (RinA and Routa1a7), all products appear to have similar band size. Since it is understood that FinC should have the larger fragments, more stringent conditions must be further pursued. The gel indicates that all samples are heterozygous for both alleles, but there was a chance that specificity is low.

Gradient PCR Results

A 60-65℃ gradient PCR was conducted on sample TM33 for external primers: (Fout_a1a7), C allele-specific primer and external: (FinC and Rout_a1a7), and A allele-specific primer and external: (RinA and Fout_a1a7). Fragments from each master-mix were stronger around the 60.0 - 61.6°C range with the exception of the external fragments exceeding 61.6℃ to 62.8℃. Any well that appears empty is a reaction that was not able to occur due to the gradient passing optimal annealing temperatures. The C and A allele-specific master-mixes appear to have a more noticeable size difference. The external are estimated to be about ~660-680 bp, C allele-specific is ~300 bp, and A allele-specific ~370-400 bp. Products indicate that sample TM33 is heterozygous.

Multiplex PCR

Results from multiplex PCR of TM33's ExoSAP products show two bands from 55-60°C. The master-mix of the PCR contained all four primers and was tested against the

gradient. These results indicate that externals and one internal (allele-specific primer) plus primer were able to successfully anneal under lower temperatures.

PCR settings were originally thought to have not been stringent for proper genotyping since they were appearing to be heterozygous. Since it is known that the DNA from all samples are mitochondrial, genetic information should only have passed on through maternal DNA, and thus sequences should have a homozygous genotype. Through ExoSAP protocols to "clean" PCR products, and undergoing a multiplex PCR to test all primers, results from **Figure 5** indicate that sample TM33 is in fact homozygous rather than heterozygous as previously stated. It is thought that because previous PCR products appeared to be heterozygous was because there was no competition between the internal primers. TM33 clearly displays two bands (one external and one internal) under a 55-65 ℃ range within the gradient rather than three bands indicating heterozygosity as seen in **Figure 3**.

Although proper functionality of the primers heavily relied on multiple PCRs, it has proven to be a low-cost alternative for genotyping. The tetra-primer PCR is convenient because it contains multiple primers to genotype for two strains of *Bd* rather than genotype for one strain through the use of just two primers. With the necessary amounts of reagents, PCR tubes, a small gel electrophoresis apparatus and a mini-PCR, the total baggage needed for the protocol should be relatively light; This will benefit scientists such as Dr. Rodriguez by providing more convenient means for sampling and genotyping rather than transporting samples cross-country.

Future directions

From this point forth, the designed tetra-primer PCR will be tested on a mini-PCR machine for the preparation of genotyping in Ecuador. It is vital to confirm genotyping accuracy before departure for there will virtually be no chance of change once in Ecuador. Along with mini-PCR prep, other samples aside from TM33 will be confirmed for homozygosity or possible heterozygosity. For future reference, estimation of better primer competition may be taken into consideration when designing primer assays through Primer1. Rather than only ordering primers under default settings, more options that affect primer annealing and expression such as salt concentration or maximum and minimum melting temperature may also be tested.

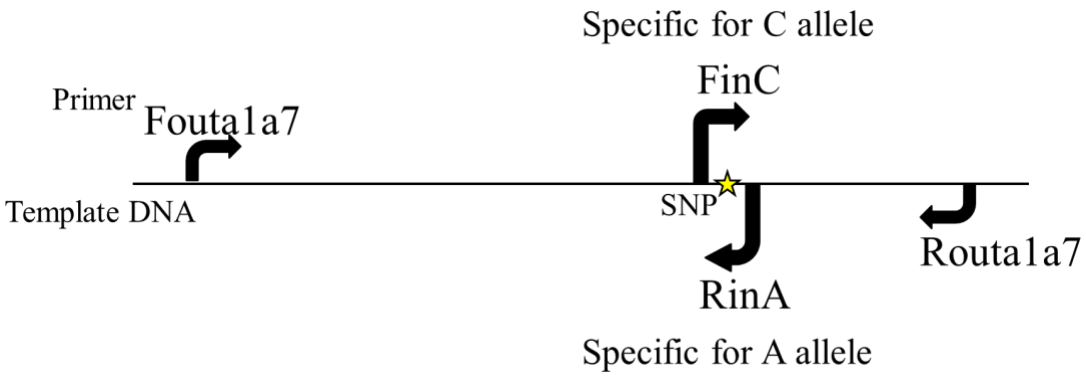

Figure 1. *Primer location in comparison to the SNP location. Although five total primers were ordered and used, Fout_a7 was no longer used after initial testing due to insufficient primer binding. Amplifications for the A allele will result in larger fragments compared to amplicons for the C allele.*

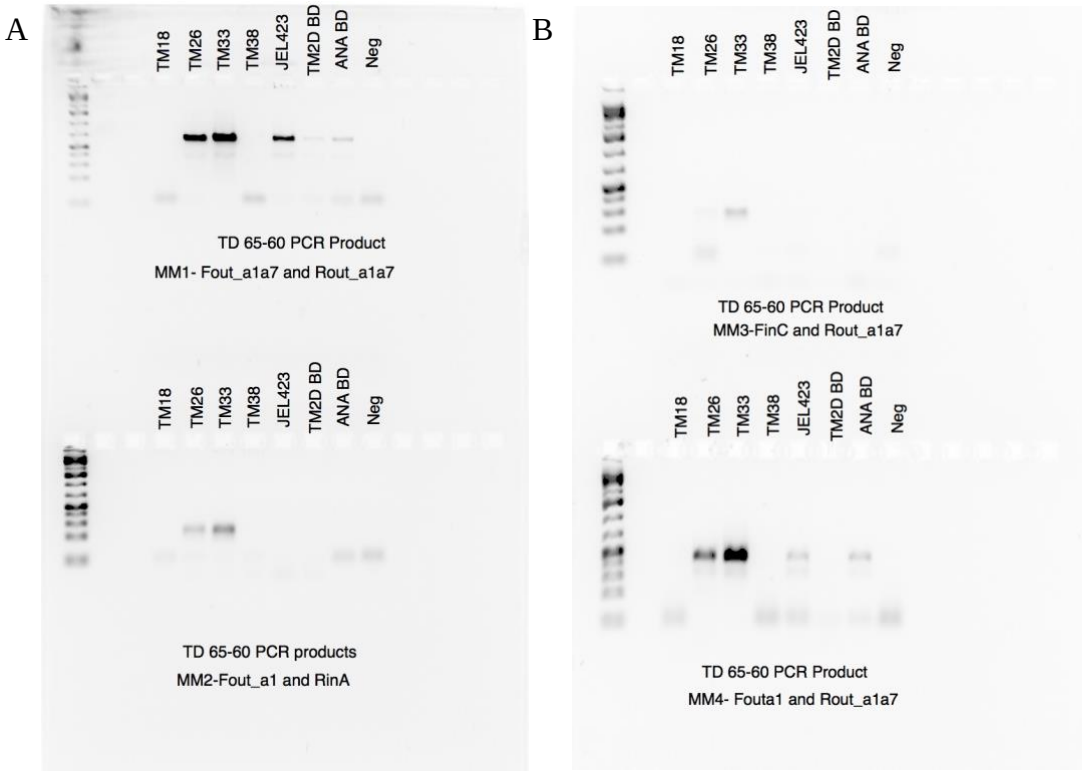

Figure 2. A) *Touchdown PCR with the temperature range: 56-60°C for mastermix 1: (Fout_a1a7 and Rout_a1a7) and mastermix 2: A allele-specific primer (RinA) and external Fout_a1.* B) *products for both mastermix 3 with C allele-specific primer (FinC) and external Rout_a1a7 while mastermix 4 displays products for the externals: Fout_a1 and Rout_a1a7.*

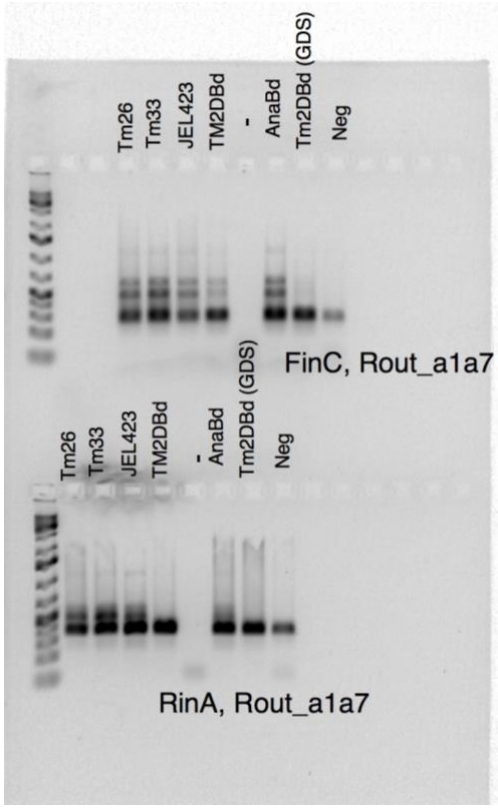

*Figure 3. Allele specific primers FinC and RinA are paired with external, reverse primer, Routa1a7. *Note: well 5 was purposely left empty.*

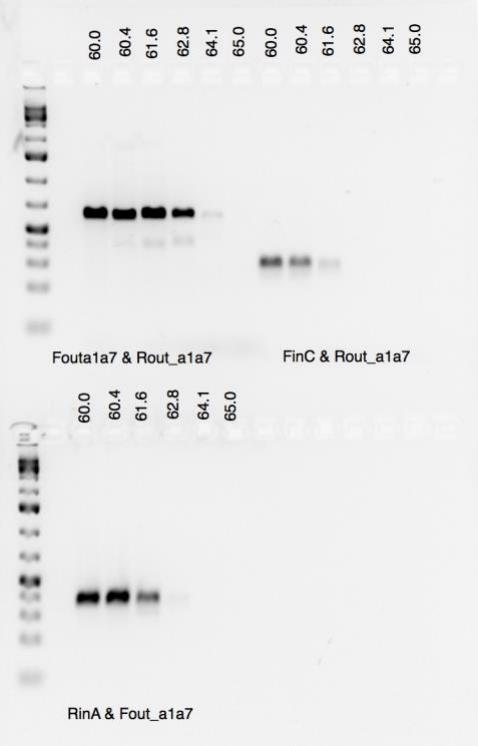

*Figure 4. Sample TM33's PCR products from a gradient PCR (60-65°C) are shown with three master-mixes externals: (Fou_ta1a7, Rout_a1a7), C allele-specific: (FinC, Rout_a1a7), and A allele-specific (RinA, Fout_a1a7). *Note: No wells were left empty, each had a reaction take place.*

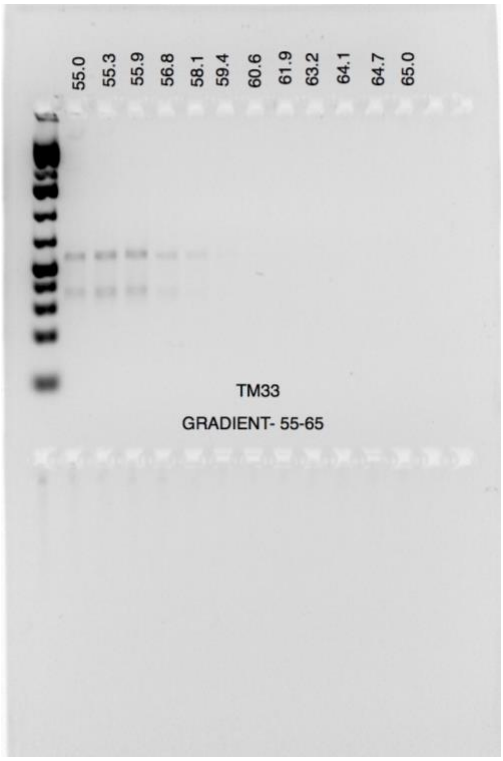

*Figure 5. ExoSAP products from sample TM33 underwent multiplex PCR at a 55-65°C gradient. Primers were only able to anneal within a 55.0-58.1°C range. *Note: extra generuler was included into the ladder's well (well 1), but should not hinder results.*

Table 1: *This table displays primer names, amounts of primer (nmol) and desired volume of EB (nuclease free H_2O) for respective primers in order to create 100µM stock solutions.*

Primer	Before Suspension (nmol)	Nuclease free H_2O volume (µL)
189057932 (Fin C)	29.80	298.0
189057933 (Rin A)	40.70	407.0
189057934 (Fout_a1)	21.80	218.0
189057935 (Rout_a1a7)	32.30	323.0
189057936 (Fout_a7)	30.10	301.0

Table 2: Above, each master mix and its components are shown along with the volumes. Forward and reverse primers are recognized by the F (forward) and R (reverse) in the beginning of the names.

Contents (µl)	Master-mix 1	Master-mix 2	Master-mix 3	Master-mix 4
Dream Taq	62.50	62.50	62.50	63.50
Forward	01.25	01.25	01.25	01.25
Primers	Fout_a1a7	Fout_a1	FinC	Fout_a1
Reverse	Rout_a1a7	Rout_a1a7	Rout_a1a7	R_outa1a7
Primers				
MgCl	05.00	05.00	05.00	05.00
BSA	01.00	01.00	01.00	01.00
Nuc. Free	56.50	56.50	56.50	56.50
H_2O				

*<u>Table 3</u>**:** For each reaction, 12.75µl of respective master mix and was transferred to each PCR 8-tube strip along with respective tDNA. This was repeated 3 more times for a total of 32 total PCR reactions. Products may be viewed on **(Figure 1)** and **(Figure 2)**.*

Reaction #	**Sample tDNA**	**Master Mix (µl)**
1	Tm18 (50µl)	12.75
2	Tm26 (50µl)	12.75
3	Tm33 (50µl)	12.75
4	Tm38 (50µl)	12.75
5	JEL423 (50µl)	12.75
6	Tm2DBd (50µl)	12.75
7	AnaBd (50µl)	12.75
8	Negative Control (50µl)	12.75

Table 4: This table shows the PCR contents of master-mix 1 and 2 along with their allele-specific primers FinC, RinA and external Rout_a1a7. Reaction volumes are shown on

Contents (µL)	Master-Mix 1	Master-Mix 2
DreamTaq	56.25	56.25
Forward	1.125- FinC	1.125- RinA
Reverse	1.125- Rout_a1a7	1.125- Rout_a1a7
$MgCl_2$	04.50	04.50
BSA	00.90	00.90
Nuclease Free H_2O	50.85	50.85

Table 5: This table displays the sample and master-mix volumes required for each reaction. For both master-mixes, there was a total of 16 reactions. ***Gel 3*** *displays these products.*

Reaction #	**Sample tDNA**	**Master Mix (µl)**
1	Tm26 (50µl)	12.75
2	Tm33 (50µl)	12.75
5	JEL423 (50µl)	12.75
6	Tm2DBd (50µl)	12.75
7	AnaBd (50µl)	12.75
8	Negative Control (50µl)	12.75

Table 6: For the gradient PCR contents are listed above with respective amounts to each master-mix tube. Sample Tm33 was used for each reaction with 12.75µL of master-mix and 0.5 of tDNA. Products may be seen on **Figure 4**.

Contents (µL)	**Mastermix 1**	**Mastermix 2**	**Mastermix 3**
DreamTaq	50.00	50.00	50.00
Forward	01.00	01.00	01.00
Reverse	01.00	01.00	01.00
$MgCl_2$	04.00	04.00	04.00
BSA	00.80	00.80	00.80
Nuclease Free H_2O	45.20	45.20	45.20

Table 7: *Contents and volumes used for the gradient multiplex PCR are shown above. Reactions contained 12.25µL of master-mix and 00.50µL of sample TM33's tDNA.*

Contents	**Master-mix (µL)**
Dreamtaq	87.50
Forward Outer	0.875
Reverse Outer	0.875
Forward Inner	0.875
Reverse Inner	0.875
Nuclease-free H_2O	80.50

Appendix I

Output generated from Primer1.

**********OUTPUT 1**********
Forward inner primer (C allele): Melting temperature
416 CAATATTATCATTTTCTATATAAGATTGTC 445 54

Reverse inner primer (A allele):
470 ATAAATATGCCTTTAGTGAACTGAAT 445 56

Forward outer primer (5' - 3'):
282 TATATGATTAAAATCTAAATACCAGAGG 309 55

Reverse outer primer (5' - 3'):
626 TCTTATCTATTACATAAACCTGAAGATC 599 55

Product size for C allele: 212
Product size for A allele: 189
Product size of two outer primers: 345

**********OUTPUT 2**********
Forward inner primer (C allele): Melting temperature
416 CAATATTATCATTTTCTATATAAGATTGTC 445 54

Reverse inner primer (A allele):
470 ATAAATATGCCTTTAGTGAACTGAAT 445 56

Forward outer primer (5' - 3'):
283 ATATGATTAAAATCTAAATACCAGAGGT 310 55

Reverse outer primer (5' - 3'):
626 TCTTATCTATTACATAAACCTGAAGATC 599 55

Product size for C allele: 212
Product size for A allele: 188
Product size of two outer primers: 344

**********OUTPUT 3**********
Forward inner primer (C allele): Melting temperature
416 CAATATTATCATTTTCTATATAAGATTGTC 445 54

Reverse inner primer (A allele):
470 ATAAATATGCCTTTAGTGAACTGAAT 445 56

Forward outer primer (5' - 3'):
237 TATTCTGGTAACCACTTAATTATTAGATT 265 55

Reverse outer primer (5' - 3'):
626 TCTTATCTATTACATAAACCTGAAGATC 599 55

Product size for C allele: 212
Product size for A allele: 234

Product size of two outer primers: 390

**********OUTPUT 4**********
Forward inner primer (C allele): Melting temperature
416 CAATATTATCATTTTCTATATAAGATTGTC 445 54

Reverse inner primer (A allele):
470 ATAAATATGCCTTTAGTGAACTGAAT 445 56

Forward outer primer (5' - 3'):
282 TATATGATTAAAATCTAAATACCAGAGGT 310 55

Reverse outer primer (5' - 3'):
626 TCTTATCTATTACATAAACCTGAAGATC 599 55

Product size for C allele: 212
Product size for A allele: 189
Product size of two outer primers: 345

**********OUTPUT 5**********
Forward inner primer (C allele): Melting temperature
416 CAATATTATCATTTTCTATATAAGATTGTC 445 54

Reverse inner primer (A allele):
470 ATAAATATGCCTTTAGTGAACTGAAT 445 56

Forward outer primer (5' - 3'):
283 ATATGATTAAAATCTAAATACCAGAGG 309 55

Reverse outer primer (5' - 3'):
626 TCTTATCTATTACATAAACCTGAAGATC 599 55

Product size for C allele: 212
Product size for A allele: 188
Product size of two outer primers: 344

**********OUTPUT 6**********
Forward inner primer (C allele): Melting temperature
416 CAATATTATCATTTTCTATATAAGATTGTC 445 54

Reverse inner primer (A allele):
470 ATAAATATGCCTTTAGTGAACTGAAT 445 56

Forward outer primer (5' - 3'):
284 TATGATTAAAATCTAAATACCAGAGGT 310 55

Reverse outer primer (5' - 3'):
626 TCTTATCTATTACATAAACCTGAAGATC 599 55

Product size for C allele: 212
Product size for A allele: 187
Product size of two outer primers: 343

**********OUTPUT 7**********
Forward inner primer (C allele): Melting temperature
416 CAATATTATCATTTTCTATATAAGATTGTC 445 54

Reverse inner primer (A allele):
470 ATAAATATGCCTTTAGTGAACTGAAT 445 56

Forward outer primer (5' - 3'):
181 ATCATTAATGAAATATATATACGTCCC 207 55

Reverse outer primer (5' - 3'):
626 TCTTATCTATTACATAAACCTGAAGATC 599 55

Product size for C allele: 212
Product size for A allele: 290
Product size of two outer primers: 446

**********OUTPUT 8**********
Forward inner primer (C allele): Melting temperature
416 CAATATTATCATTTTCTATATAAGATTGTC 445 54

Reverse inner primer (A allele):
470 ATAAATATGCCTTTAGTGAACTGAAT 445 56

Forward outer primer (5' - 3'):
181 ATCATTAATGAAATATATATACGTCCC 207 55

Reverse outer primer (5' - 3'):
626 TCTTATCTATTACATAAACCTGAAGATC 599 55

Product size for C allele: 212
Product size for A allele: 290
Product size of two outer primers: 446

**********OUTPUT 9**********
Forward inner primer (C allele): Melting temperature
416 CAATATTATCATTTTCTATATAAGATTGTC 445 54

Reverse inner primer (A allele):
470 ATAAATATGCCTTTAGTGAACTGAAT 445 56

Forward outer primer (5' - 3'):
279 TCTTATATGATTAAAATCTAAATACCAGAG 308 55

Reverse outer primer (5' - 3'):
626 TCTTATCTATTACATAAACCTGAAGATC 599 55

Product size for C allele: 212
Product size for A allele: 192
Product size of two outer primers: 348

**********OUTPUT 10**********
Forward inner primer (C allele): Melting temperature

416 CAATATTATCATTTTCTATATAAGATTGTC 445 54

Reverse inner primer (A allele):
470 ATAAATATGCCTTTAGTGAACTGAAT 445 56

Forward outer primer (5' - 3'):
177 AGTTATCATTAATGAAATATATATACGTCC 206 55

Reverse outer primer (5' - 3'):
626 TCTTATCTATTACATAAACCTGAAGATC 599 55

Product size for C allele: 212
Product size for A allele: 294
Product size of two outer primers: 450

IV. References

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